

Antitumor and Antioxidant Effect of Celastrol Isolated from *Celastrus punctatus*

Vei-Juh Lee¹ Yuan-Shiun Chang^{1,2} Ming-Tsuen Hsieh¹ Li-Kang Ho³

¹*Institute of Chinese Pharmaceutical Sciences, China Medical College*

²*Chinese Crude Drug Pharmacy, China Medical College Hospital*

³*Department of Pharmacology, National Yang-Ming University*

Abstract

Celastrus punctatus (CP) is a renowned folk medicine for the treatment of rheumatism, malaria and other febrile diseases in Taiwan. Extract from plants of the Celastraceae family has been used as a commercial preparation for the treatment of cancer. The purpose of this study is to examine the anti-tumor effect of *Celastrus punctatus*.

Methanolic extracts obtained from the root of CP were separated by using fractionation chromatography and divided into five fractions: *n*-hexane, chloroform, ethyl-acetate, butanol and water layer (Fr. A-Fr. E). The ethyl-acetate and chloroform layer exerted strong anti-cancerous effect against five cancer cell lines including KB, P-388, HT-29, A-549, and HL-60. The *n*-hexane and water layer exerted a cytotoxic effect on P-388 and HL-60, whilst the butanol layer affected only KB cells. In addition, a red crystal, which exerted a strong inhibiting effect on all five cancer cell lines, was isolated from the ethyl-acetate layer and identified as celastrol. The anti-tumor effect of ethyl acetate layer might be originated from the bio-active compound, celastrol. As there were increasing evidences indicating that reactive oxygen species (ROS) might play an important role in the pathogenesis of cancer, the anti-tumor effect of celastrol might be attributed to the inhibition of superoxide production induced by PMA and/or the scavenge of free radicals.

Key words: *Celastrus punctatus*, Celastrol, Anti-cancer, Antioxidant

Corresponding to : Yuan-Shiun Chang

Address : Institute of Chinese Pharmaceutical Sciences, China Medical College, 91, Hsueh Shih Road, Taichung, Taiwan, R.O.C.

Introduction

Celastrus punctatus Thunb (CP, Celastraceae family) is a famous folk medicine in Taiwan⁽¹⁾. The root of CP is used to stimulate blood circula-

tion and treat swelling of unknown sources as described in "Chih-Wu-Ming-Shih-Tu-Kao" of Ching Dynasty(1848 A.D.)⁽²⁾. Besides, among the plants of Celastraceae family, Tripterygium⁽³⁾ and Maytenus⁽⁴⁾ were found to have antitumor activity. As a member of Celastraceae family, *Celastrus* specie is assumed to be effective against tumor cell lines. In the present study, the fractionated extracts of the roots of CP were examined for the antitumor activity. The bioactive component of CP was also isolated⁽⁵⁾. In our present study, we isolated a red compound and the structure was identified by MS and NMR spectra as celastrol. Celastrol possesses a phenolic -OH group, a carboxyl group, and a quinoid moiety in its triterpene structure(Fig. 1). Among all the triterpenes, celastrol has been isolated from Celastraceae family as an active constituent against lipid peroxidation⁽⁶⁾, but the antitumor effect of celastrol had never been reported.

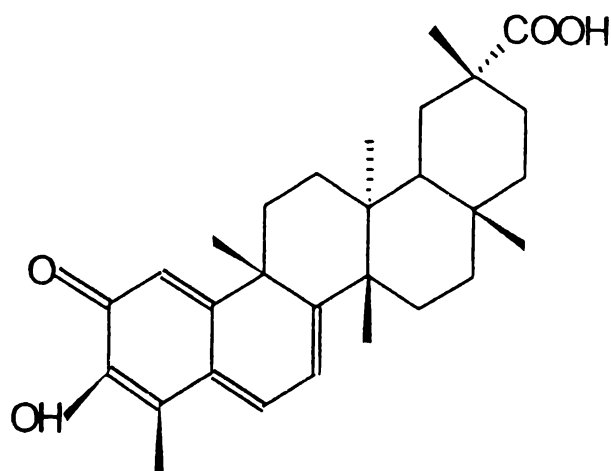


Fig. 1: Chemical structure of celastrol

Reactive oxygen species(ROS) are implicated in the pathogenesis of a wide variety of human diseases including cancer⁽⁷⁾⁽⁸⁾. The induction of oxidative stress in the target tissue had been proposed as a possible mechanism of action during carcinogenesis. In parallel to the antitumor effect of celastrol, the superoxide scavenging effect was also examined. This ROS quenching action may involved in the antitumor effect of celastrol.

Materials and Methods

Preparation of roots of *Celastrus punctatus*.

The plant used in current study was identified as root of *Celastrus punctatus*. The roots were sliced into small pieces and covered with methanol under room temperature for a week and repeated for three times, filtered, and concentrated. The concentrated extract was then resuspended in water for further fractionation extraction by *n*-hexane, chloroform, ethyl-acetate and butanol. Celastrol was isolated from the EtOAc layer as described in our previous report.

Materials

n-Hexane, chloroform, ethyl-acetate, butanol, and methanol were purchased from ALPS Co. (Taiwan). The medium used for tumor cell lines and lucigenin were all from Sigma(St Louis, MO, USA).

Cell culture

There are 5 tumor cell lines(KB, P-388, A-549, HT-29, HL-60) used for antitumor assay. KB cell was cultured with DMEM containing 10% FBS. P-388 cell was cultured with Fischer's medium containing 10% FBS. A-549 cell was cultured with RPMI-1640 containing 10% FBS, 2mM glutamine, 1 mM sodium pyruvate and 50 mM mercaptoethanol. HT-29 cell was cultured with alpha MEM containing 10% FBS. HL-60 cell was cultured with RPPMI 1640 containing 15% FBS.

Free radical scavenging effect was performed by using human umbilical vein endothelial cells (HUVECs). HUVECs were isolated from human umbilical cord as previously described⁽⁹⁾. HUVECs were grown in petri dishes and passaged for one time by 1.0 % trypsin solution (Gibco) until the monolayer became confluent. The culture medium was changed into medium 199 containing only 2% fetal calf serum(FCS), and

the cells were incubated overnight prior to the experiment.

Antitumor Assay(MTT method)

The antitumor study was performed by MTT(3-(4,5 dimethylthiazol-2,5-diphenyltetrazolium bromide) method⁽¹⁰⁾. MTT assay was performed by suspending the tumor cell lines in RPMI 1640+ 10% FBS, pouring 180 μ l(containing 25,000 cells) each into the wells of a 96-well culture plate adding the antitumor extracts as a final concentration of 0.5, 5, and 50 μ g/ml, and culturing in an incubator at 37°C for 48 hrs. After culturing in MTT assay, antitumor agents were washed out with PBS. Then 20 μ l(2 mg/ml) of MTT reagent(MTT 4 mg/ml+ 0.1 M sodium succinic acid) was added and incubated at 37°C for 4 hrs. The formazan formed was extracted with 150 μ l dimethyl sulfoxide and measured at a wavelength of 540 nm with ELISA reader and calculating the inhibition index. According to the American NCI rules, crude extracts are assumed to have antitumor effect with $ED_{50} \leq 20$ μ g/ml. A pure compound is assumed to have antitumor effect with $ED_{50} \leq 4$ μ g/ml.

Measurement of superoxide production

Superoxide production was monitored by chemiluminescence of lucigenin(bis-N-methylacridinium nitrate)⁽¹¹⁾. Briefly, ECs were lysed immediately after phorbol 12-myristate 13-acetate(PMA, 5 μ g/ml) treatment for 5 minutes with a lysis buffer containing lucigenin(200 μ M/L). Photons were counted for 6 seconds immediately for 5 cycles by using a microplate scintillation counter (Topcount, Packard Instrument Co. Meriden, CT). Samples with superoxide dismutase (SOD) were used as blank control. The average single photon count was recorded.

Measurement of lipid peroxidation.

Using an in vitro cell-free system, low-den-

sity lipoprotein(LDL, 50 μ g/ml) was oxidized with $CuSO_4$ for 24 hours by pretreatment with or without celastrol. The extent of oxidation of the LDL preparations was measured by the thiobarbituric acid reactivity assay in microtiter-plate⁽¹²⁾. Malondialdehyde (MDA) was used as a standard, and the thiobarbituric acid-reactive substances (TBARS) were recorded as equivalents of MDA per mg of LDL proteins.

Statistical Analysis

Statistical analyses were performed by using the Student's t-test. Data are expressed as mean+ SE. Statistical significance was defined as $P < 0.05$.

Results

Antitumor effect of fractionated extracts of CP

CP subfractions showed different inhibition strength on the five cancer cell lines(KB, P-388, A-549, HT-29, HL-60) as showing in Table 1. Fr.B and Fr.C showed the strongest antitumor effect. The other fractions also have suppression action on parts of this five cancer cell lines. It seemed that extracts of CP indeed had antitumor effects.

Antitumor effect of celastrol

Celastrol was isolated from the EtOAc layer by using silica gel and Sephadex LH-20. It is a red crystal as identified as a triterpene. Celastrol strongly inhibited all the five cancer cell lines as shown in Table 2.

Effect of celastrol on PMA-induced superoxide generation

Phorbol ester was shown to have tumor promotion effect and generation of superoxide⁽¹³⁾. PMA(5 μ g/ml), a phorbol ester⁽¹⁴⁾, was selected to evaluate the effect of celastrol on changing of intracellular ROS levels in endothelial cells as shown in Fig.2. PMA induced about 5 time increase

of intracellular superoxide generation monitored by the lucigenin-amplified chemi-luminescence. Pretreatment of celastrol (10 μ M) totally inhibited the PMA-induced generation of superoxide.

Table 1、ED₅₀ value (μ g / ml) of antitumor effect from CP subfractions

Agent	KB	P-388	A-549	HT-29	HL-60
Fr.A	750.00	8.64*	>50.00	27.87	8.20*
Fr.B	9.53*	2.17*	10.94*	6.08*	1.90*
Fr.C	13.81*	4.54*	11.62*	9.52*	2.15*
Fr.D	750.00	10.82*	17.80*	12.38*	7.73*
Fr.E	750.00	15.20*	>850.00	>50.00	2.16*

1. Data are shown as mean (n=8)

2. If ED₅₀ $\leq$ 20 μ g/ml, it has antitumor effect*.

3. Fr.A : Hexane layer; Fr.B : Chloroform layer; Fr.C : Ethyl-acetate layer

Fr.D : Butanol layer; Fr.E : Water layer

Table 2、ED₅₀ value (μ g / ml) of antitumor effect of celastrol

Agent	KB	P-388	A-549	HT-29	HL-60
Celastrol	0.63*	0.13*	0.28*	0.53*	0.24*

* If ED₅₀ $\leq$ 4 μ g/ml, it has antitumor effect.

Effect of celastrol on lipid peroxidation

Cu²⁺/O₂ induced lipid peroxidation of human LDL system was used for selection of bioactive antioxidants. LDL(50 μ g) could oxidize by CuSO₄ by measuring with TBARS value as shown in Fig. 3. The TBARS value (equivalents of MDA per mg LDL) was about 3.07. After 24 hours, LDL was oxidized naturally to a TBARS value of 5.05, if pre-incubation of CuSO₄ the value increased to 8.5. Pretreatment of celastrol reduced the TBARS value from 8.5 to 6.64 (P<0.05). Here, we concluded that celastrol could inhibit the oxidation of LDL.

Discussion

Celastrus punctatus(CP) is a famous folk medicine used in Taiwan for the treatment of rheumatic arthritis⁽¹⁾. The pharmacological effects of Celastraceae family were well defined as anti-viral⁽¹⁵⁾, anti-fatigue⁽¹⁶⁾, and anti-bacterial⁽¹⁷⁾

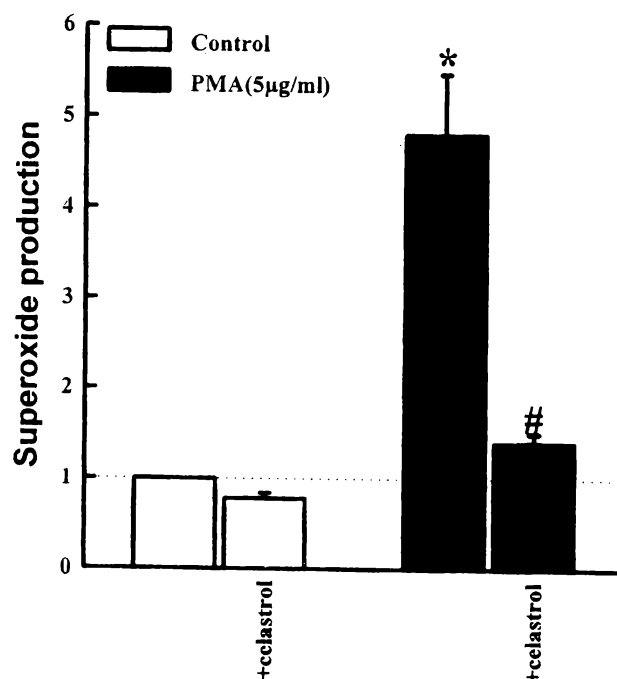


Fig. 1: Effect of celastrol on PMA-generated superoxide radicals

Endothelial cells were grown on petri dishes. PMA was treated for 5 minutes to generate superoxide radicals. In experimental groups, celastrol was pretreated for 30 minutes. (Data are shown as mean+SE, n=6; *P<0.05 vs control; # P<0.05 vs PMA)

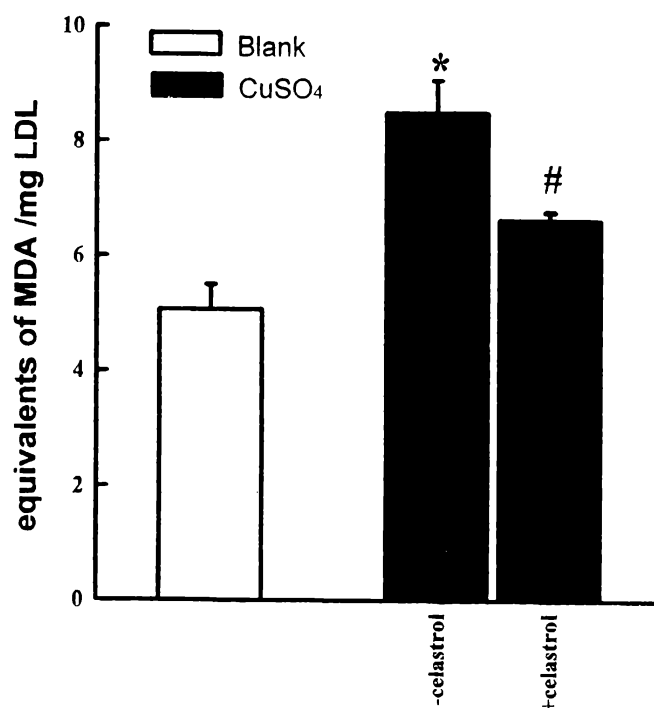


Fig. 2: Effects of celastrol on CuSO₄-induced lipid peroxidation

LDL(50mg) was oxidized with CuSO₄ for 24 hours with or without pretreatment of celastrol for 30 minutes. (Data are shown as mean+SE, n=6; *P<0.05 vs blank; # P<0.05 vs -celastrol)

activities. In the twentieth century, fatal effect of cancer makes people to concern the pathogenesis of cancer. Among the plants of Celastraceae family, *Tripterygium*⁽³⁾ and *Maytenus*⁽⁴⁾ were found to have antitumor activity. As a member of Celastraceae family, *Celastrus* is assumed to be effective against tumors. In the present study, the fractionated extracts of the roots of CP were examined for the antitumor activity. The antitumor effect was evaluated by using MTT method with five cancer cell lines KB, P-388, A-549, HT-29, HL-60. Fractionated extractions of CP showed strong inhibition activity against the cancer cells especially ethyl-acetate and chloroform layers. It is noticed that the lipid solubility is an important factor affecting the absorption and utilization of antitumor drugs. The antitumor effect of crude extract of CP distributed in the non-polar layers seemed to be reasonable. Besides, what is the bioactive component in CP corresponding to the antitumor effect was an interesting subject in our research. A red compound was isolated from the ethyl-acetate layer which composed the strongest antitumor action. After elucidated with MS and NMR spectra, the structure of the red crystal was identified to be celastrol. Celastrol also showed strong antitumor effect which means that celastrol might corresponding for the antitumor effect of ethyl-acetate layer.

Cell growth and differentiation are regulated by influences from both within and without the cell. In cancer cells, key components of the signaling processes are often subverted by oncogenes and resulting in unregulated signaling. Oncogene products include growth factors, growth factor receptors, the signal transducers, and transcription factors⁽¹⁸⁾. Recent evidence suggested that at moderately concentrations, certain forms of reactive oxygen species (ROS) may act as signal transduction messengers⁽⁷⁾. There were already proofs indicating that ROS involved in oncogene products regulation. Thus ROS may play an important role in the cellular signaling. Reactive oxygen species(ROS) such as hydrogen perox-

ide, superoxide and hydroxyl radical, are implicated in the pathogenesis of a wide variety of human disease including vascular diseases and cancer. The induction of oxidative stress in the target tissues had been proposed as a possible mechanism of action during carcinogenesis. Among all of the potential carcinogens, ROS were also thought to be involved in the development of different types of human cancer. ROS have been implicated in a multistage carcinogenic process, including oxidative DNA damage⁽¹⁹⁾, inhibition of DNA repair⁽²⁰⁾, tumor initiation and promotion⁽²¹⁾. In accord with the antitumor effect of celastrol, whether or not celastrol was involved in the regulation of ROS generation was also examined.

Studies have been shown that phorbol ester such as PMA was a tumor promotor and could increase intracellular superoxide. Thus, PMA was chosen to activate the generation of superoxide for assay of antioxidant effect of celastrol. Besides, LDL oxidation by $\text{Cu}^{2+}/\text{O}_2$ is a common method in screening for antioxidants⁽²²⁾. Oxidized LDL obtained from incubation with $\text{Cu}^{2+}/\text{O}_2$ exhibits biological, immunological, and physicochemical properties which are remarkably similar to those of in vivo lipid peroxidation⁽²³⁾. Furthermore, in cancer cells, there were changes in the physical state of lipid peroxidation due to the disturbance in the metabolism of ROS⁽²⁴⁾. For these reasons, the in vitro method using $\text{Cu}^{2+}/\text{O}_2$ -induced LDL oxidation was selected to evaluate the antioxidant activity of celastrol. In our present study, celastrol was shown to have strong superoxide radical quenching activity and anti-lipid peroxidation effect. These findings were similar to other reports. Previously studies⁽⁶⁾ indicated that celastrol was a potent inhibitor on mitochondria lipid peroxidation; its 50% inhibitory concentration is about 7 μM . Celastrol had direct radical scavenging actions and prevention of attack of active oxygen complexes on the membrane. The dienone-phenol moiety is responsible for the direct radical scavenging, and the anionic carboxyl group is important for retardation of the initiation

of free radical chain reactions.

In conclusion, our present study indicated that *Celastrus punctatus* showed a strong antitumor action especially in ethyl-acetate and chloroform layer. Celastrol, isolated from ethyl-acetate layer, might be a bioactive component corresponding for the tumor suppression effect. However, the bioactive constituents of the chloroform layer remains for further study. Besides, celastrol was effectively inhibited the generation of superoxide radicals and lipid peroxidation. This antioxidant ability might implicated in the antitumor activity.

Acknowledgment

We thank Dr. Chang-Yih Duh, Department of Marine Resources of National Sun Yat-Sen University for providing the facilities of MTT assay and National Science Council, R.O.C. for grant support (NSC-82-0420-B-039-017-M13).

References

1. Kan WS: Manual of Medicinal Plants in Taiwan Vol 1-3, National Research Institute of Chinese Medicine, Taipei, pp470-471, 1985.
2. 吳其濬: 植物名實圖考, 上卷, 卷十九, 蔓草類, 475 頁, 世界書局(楊家駱編)。
3. 劉昌孝: 具有抗癌活性之新天然物, 第四期, 48 頁, 中草藥通訊, 1976。
4. 吳征縊、周太炎、肖培根: 新華本草綱要(一), 295-296 頁, 上海科學技術出版社, 1988。
5. Lee VJ, Ho LK, Hsieh MT and Chang YS: Studies of the constituents of the roots of *Celastrus punctatus*, J Chinese Medicine, in press.
6. Sassa H, Kogure K, Takaishi Y, and Terada H: Structural basis of potent antioxidation activity of the triterpene celastrol in mitochondria effect of negative membrane surface charge on lipid peroxidation. Free Radic Biol Med 17(3): 201-207, 1994.
7. Sen CK and Packer L: Antioxidant and redox regulation of gene transcription. FASEB J 19 (7):709-720, 1996.
8. Matsubara T and Ziff M: Superoxide anion release from human endothelial cells: synergism between a phorbol ester and a calcium ionophore, J Cell Physiol 127:207-210, 1986.
9. Gimbrone MA: Culture of vascular endothelium. In: Progress in haemostasis and thrombosis. T.H. Spaet, ed. Grune and Stratton. NY, 3:1-28, 1976.
10. Denizot F and Lang R: Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods, 89:271-277, 1986
11. Gyllenhammer H: Lucigenin chemiluminescence in the assessment on neutrophil superoxide production. J Immunol Methods 97: 209-213, 1987.
12. Yue TL, Cheng HY, Lysko PG, McKenna PJ, Feuerstein R, Gu JL, Lysko KA, Davis LL, and Feuerstein G: Carvedilol, a new vasodilator and beta adrenoreceptor antagonist, is an antioxidant and free radical scavenger. J Pharmacol Exp Ther 263:92-98, 1992.
13. Witz G: Active oxygen species as factors in multistage carcinogenesis. Proc Soc Exp Biol Med 198(2):675-682, 1991.
14. Marks F and Gschwendt M: Protein kinase C and skin tumor promotion. Mutat Res 333 (1-2):161-172, 1995.
15. Sengupta A, Sengupta C, and Mazumder UK: Chemical investigations of *Celastrus paniculatus* seed oil. Food Wiss Technol 89(3): 119-223, 1987.
16. Willaman JJ and Schubert BG: Alkaloids bearing plants and their contained alkaloids. Ars Usda Tech Bull 1234, 1961.
17. Dabral PK and Sharma RK: Evaluation of role of rumalaya and geriforte in chronic arthritis-A preliminary study. Probe 22(2): 120-127, 1982.
18. Cantly LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, and Soltoff S: Oncogenes and signal transduction. Cell 64: 281-302, 1991.
19. Wagner JR, Hu CC, and Ames BN: Endogenous oxidative damage of deoxycystidine in DNA. Proc Natl Acad Sci USA 89 (3380-3384, 1992.
20. Hu JJ, Dubin N, Kurland D, Ma BL, and Routh GC: The effects of hydrogen peroxide on DNA repair activities. Mutat Res 336:

193-201,1995.

21. Goldstein BA and Witz G: Free radicals and carcinogenesis. *Free Radic Res Commun* 11: 3-10,1990.
22. Mao SJ, Yates MT, Parker RA, Chi EM, and Jackson RL: Attenuation of atherosclerosis in a modified strain of hypercholesterolemic Watanabe rabbits with use of a probucol analogue(MDL29,311) that does not lower serum cholesterol. *Arterioscler Thromb* 11(5):

1266-1275,1991.

23. Yia-Herttuala S, Palinski W, Rosenfield M, Parathasarathy S, Carew TE, Bulter S, Witztum J, and Steinberg D: Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 84:1086-1095,1989.
24. Masotti L, Casali E, and Galeotti T: Lipid peroxidation in tumor cells. *Free Radic Biol Med* 4(6):377-386,1988.

南蛇醇(Celastrol) 抗癌及抗氧化作用之研究

李威著¹ 張永勳^{1,2} 謝明村¹ 何禮剛³

¹中國醫藥學院 中國藥學研究所

²中國醫藥學院附設醫院 藥劑部中藥局

³國立陽明大學 藥理學科暨研究所

摘 要

光果南蛇藤(*Celastrus punctatus*)是台灣著名民間藥，「大鐵牛入石」的來源植物之一，民間一般襲用其治療風濕關節腫痛、腰痛、經閉等。此外，在衛矛科植物中已有治療癌症之製劑上市，而光果南蛇藤身為衛矛科之一員，故本實驗針對其抗癌之作用加以研究。經由不同溶媒抽提及濃縮後，共得到五個部分之抽提物，分別為正己烷，氯仿，乙酸乙酯，正丁醇及水層，各層之抽出物利用 MTT 法進行抗癌試驗，發現南蛇藤之各層抽出物對於所使用之五種癌細胞(KB, P-388, A-549, HT-29, HL-60)均有不同程度之抑制作用，其中以乙酸乙酯層及氯仿層之抗癌效果最好。同時在乙酸乙酯層中分離出一個紅色之化合物，經由光譜分析及文獻之比對後，證明此成分為南蛇醇(celastrol)。同樣的，南蛇醇亦具有良好的抗癌作用，而且可能是乙酸乙酯層中之抗癌活性成分。而愈來愈多證據顯示癌症之產生與活性氧族群(Reactive oxygen species)有關，本實驗利用 PMA 來產生過多的 superoxide radical，若事先以南蛇醇處理，則會抑制 PMA 所誘生之 superoxide radical 產生。同樣的，在脂質過氧化的實驗中，南蛇醇亦有防止低密度脂蛋白氧化之作用。值得注意的是 PMA 本身為具有促進腫瘤細胞之作用，故而南蛇醇之抗氧化劑作用可能與其對抗癌細胞之作用有相關聯。

關鍵詞：南蛇醇、光果南蛇藤、抗癌、抗氧化